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Physical and Functional Association of Human Protein *O*-Mannosyltransferases 1 and 2*

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Keiko Akasaka-Manya[‡], Hiroshi Manya[‡], Ai Nakajima[§], Masao Kawakita[§], and Tamao Endo[‡1]

From the [‡]Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, Tokyo 173-0015, Japan and [§]Department of Applied Chemistry, Kogakuin University, Tokyo 163-8677, Japan

A defect of protein *O*-mannosylation causes congenital muscular dystrophy with brain malformation and structural eye abnormalities, so-called Walker-Warburg syndrome. Protein *O*-mannosylation is catalyzed by protein *O*-mannosyltransferase 1 (POMT1) and its homologue, POMT2. Coexpression of POMT1 and POMT2 is required to show *O*-mannosylation activity. Here we have shown that POMT1 forms a complex with POMT2 and the complex possesses protein *O*-mannosyltransferase activity. Results indicate that POMT1 and POMT2 associate physically and functionally *in vivo*. Recently, three mutations were reported in the *POMT1* gene of patients who showed milder phenotypes than typical Walker-Warburg syndrome. We coexpressed these mutant POMT1s with POMT2 and found that none of them had any activity. However, all POMT1 mutants, including previously identified POMT1 mutants, coprecipitated with POMT2. These results indicate that the mutant POMT1s could form heterocomplexes with POMT2 but that such complexes are insufficient for enzymatic activity.

Dystrophin-glycoprotein complex is composed of α-, β-dystroglycan (DG),[2] dystrophin, and some other molecules. Dystrophin-glycoprotein complex is thought to act as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton (1). α-DG is a central component of the dystrophin-glycoprotein complex and is heavily glycosylated, and its sugars have a role in binding to extracellular matrixes such as laminin, neurexin, and agrin (2). Previously we reported that the glycans of α-DG include *O*-mannosyl oligosaccharides and that a sialyl *O*-mannosyl glycan, Siaα2–3Galβ1–4GlcNAcβ1–2Man, is a laminin binding ligand of α-DG (3). We have also found that muscle-eye-brain disease (OMIM 253280), a congenital muscular dystrophy, was caused by mutations in the gene encoding POMGnT1 (protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1), which forms a GlcNAcβ1–2Man linkage of *O*-mannosyl glycans (4, 5).

Protein *O*-mannosyltransferase 1 (POMT1) and its homologue POMT2 are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis (6). Mutations in the *POMT1* and *POMT2* genes are considered to be the cause of Walker-Warburg syndrome (WWS: OMIM 236670), an autosomal recessive developmental disorder associated with congenital muscular dystrophy, neuronal migration defects, and ocular abnormalities (7, 8). Previously, seven mutations in the *POMT1* gene (G76R, Q303X, Q385X, L421del, V428D, V703fs, and G722fs) were identified in patients with WWS (7, 9). We have demonstrated that these mutations in the *POMT1* gene lead to defects of POMT activity (10). This may cause a defect in α-DG glycosylation and result in failure of binding to laminin or other molecules in the extracellular matrix and interrupt normal muscular function and migration of neurons in developing brain. Recently, other mutations in the *POMT1* gene were found (11–13). Among them, three mutations (G65R, A200P, and M582C) display milder pathology than typical WWS. Patients with A200P mutation are characterized by mild mental retardation and microcephaly without brain malformation (14), and patients with G65R and M582C mutations are characterized by calf hypertrophy, microcephaly, and severe mental retardation, but no eye abnormalities (15). These findings suggested that these mutations would not completely abolish enzymatic activity, which prompted us to examine POMT activities of mutated POMT1 with milder phenotypes.

PMT, protein *O*-mannosyltransferase, is evolutionarily conserved from prokaryotes, such as *Mycobacterium tuberculosis*, to eukaryotes, such as yeast, *Drosophila*, mouse, and human (16–19). In yeast *Saccharomyces cerevisiae*, *O*-mannosylation is required for the stability, correct localization, and/or function of proteins. Yeast *O*-mannosylation is initiated in the lumen of the endoplasmic reticulum (ER) by a family of PMTs that catalyze the transfer of a mannosyl residue from dolichol phosphate mannose to Ser/Thr residues of proteins (16). *S. cerevisiae* has seven PMT homologues (Pmt1p-7p) that share almost identical hydropathy profiles. The hydropathy profiles predict that PMTs are integral membrane proteins with multiple transmembrane domains (16, 20–22). The PMT family is

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[1] To whom correspondence should be addressed: Glycobiology Research Group, Tokyo Metropolitan Inst. of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015, Japan. Tel.: 81-3-3964-3241 (ext. 3080); Fax: 81-3-3579-4776; E-mail: endo@tmig.or.jp.

[2] The abbreviations used are: α-DG, α-dystroglycan; ER, endoplasmic reticulum; HEK, human embryonic kidney; WWS, Walker-Warburg syndrome; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid; PMT, protein *O*-mannosyltransferase; POMT, protein *O*-mannosyltransferase.

classified phylogenetically into the PMT1, PMT2, and PMT4 subfamilies. Members of the PMT1 subfamily (Pmt1p and Pmt5p) interact heterophilically with those of the PMT2 subfamily (Pmt2p and Pmt3p), whereas the single member of the PMT4 subfamily (Pmt4p) acts as a homophilic complex (17, 23). Although Pmt1p-4p and Pmt6p have *O*-mannosyltransferase activity by themselves (23), complex formation is essential for maximal transferase activity of yeast PMT family members (17, 24).

In human, transferase activity may also require formation of a heterocomplex of POMT1 and POMT2, because cotransfection of *POMT1* and *POMT2* up-regulates POMT activity in human embryonic kidney (HEK) 293T cells whereas expression of only one of these proteins does not (6). However, no direct evidence for a physical interaction between POMT1 and POMT2 proteins has been obtained so far. Here, we have demonstrated that POMT1 and POMT2 form a functional complex *in vivo* using immunoprecipitating techniques. Furthermore, we showed that the mutations of POMT1 protein found in WWS patients do not prevent complex formation with POMT2 but they do abolish activity of the complex.

EXPERIMENTAL PROCEDURES

Vector Construction of POMT1 Mutants and POMT2—Human *POMT1* cDNA was used for site-directed mutagenesis and was cloned into pcDNA 3.1 (Invitrogen) as described previously (6). For each of the three mutations (G65R, A200P, M582C) examined in this study, the *POMT1* gene was modified with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions as described previously (10). The three mutants were generated with the following primer pairs: G65R, 5′-CTTCTTGGATGACAGTAGGCCGCCATTTGGCC-3′ and 5′-GGCCAAATGGCGGCCTACTGTCATCCAAGAAG-3′; A200P, 5′-GTCGCTTGTTCCTGTCCAGTGGGCATCAAG-3′ and 5′-CTTGATGCCCACTGGACAGGAACAAGCGAC-3′; M582C, 5′-CAATATTGCCTACTGCCTGCACCCCAGGAC-3′ and 5′-GTCCTGGGGTGCAGGCAGTAGGCAATATTG-3′. All mutant clones were sequenced to confirm the presence of the mutations. Other mutants (G76R, L421del, V428D) and human *POMT2* cDNA were obtained as described previously (10).

Detergents—CHAPS, CHAPSO, *N,N*-bis(3-D-gluconamidopropyl)cholamide (BIGCHAP), *N,N*-bis(3-D-gluconamidopropyl)deoxycholamide (deoxy-BIGCHAP), *n*-octyl-β-D-glucoside, *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-thioglucoside, *n*-dodecyl-β-D-maltoside, *n*-octanoyl-*N*-methylglucamide (MEGA-8), *n*-nonanoyl-*N*-methylglucamide (MEGA-9), *n*-decanoyl-*N*-methylglucamide (MEGA-10), β-D-fructopyranosyl-α-D-glucopyranoside monodecanoate (SM-1000), β-D-fructopyranosyl-α-D-glucopyranoside monododecanoate (SM-1200), sodium cholate, sodium deoxycholate were purchased from Dojindo (Kumamoto, Japan), and digitonin and Triton X-100 were from Nacalai Tesque (Kyoto, Japan).

Cell Solubilization—HEK293T cells or transfected HEK293T cells were homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol with protease inhibitor mixture (3 μg/ml of pepstatin A, 1 μg/ml of leupeptin, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 900 × *g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000 × *g* for 1 h. Protein concentration was determined by BCA assay. Microsomal fractions thus obtained were solubilized with buffer (20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA) containing detergents at different concentrations at 4 °C. The solubilized microsomal membrane fractions thus obtained were subjected to POMT activity assay and Western blot analysis.

Expression of POMT1 with POMT2—Expression plasmids were transfected into HEK293T cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 3 days at 37 °C to produce POMT1 and POMT2 proteins.

Immunocytochemical Analysis—The expression vectors encoding Myc-tagged POMT1 and FLAG-tagged POMT2 were transfected into the HEK293T cells using Lipofectamine Plus reagent. Transfected HEK293T cells were grown on poly-D-lysine-coated culture slides (BD Biosciences), fixed with 4% formaldehyde for 30 min, and then permeabilized with cold methanol. After treatment with 1% bovine serum albumin in PBS at room temperature for 1 h, cells were incubated with anti-FLAG, anti-Myc (9E10), and anti-calreticulin (Santa Cruz Biotechnology, Santa Cruz, CA) for overnight at 4 °C. Next, cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 546-conjugated anti-goat IgG (Molecular Probes, Eugene, OR) for 1 h at room temperature. After a final wash with PBS, cells were observed, using fluorescence microscopy. We also used the following fluorescent primary antibodies: fluorescein isothiocyanate-conjugated anti-FLAG (Sigma) and TRITC-conjugated anti-Myc (Santa Cruz Biotechnology).

Immunoprecipitation—Microsomal fractions were lysed with assay buffer (20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside) in a final concentration of 2 mg/ml for 5 h at 4 °C. After solubilization, proteins were subjected to centrifugation at 10,000 × *g* for 30 min and precleaned with CL-6B-Sepharose (Sigma). Precleaned supernatants were mixed with anti-Myc (9E10)-agarose conjugate (Santa Cruz Biotechnology) and incubated overnight. After three washes with the assay buffer, the agarose beads were suspended in sample buffer. Samples were subjected to Western blot analysis. To assay POMT activity, the precipitated beads were suspended in 60 μl of assay buffer and used as the enzyme source.

Western Blot Analysis—The microsomal fractions (20 μg) or immunoprecipitated samples were separated by SDS-PAGE (7.5% gel), and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked in PBS containing 5% skim milk and 0.05% Tween 20, incubated with anti-POMT1 or anti-POMT2 polyclonal antibody (6) or anti-Myc (A-14) antibody (Santa Cruz Biotechnology), and treated with anti-rabbit IgG conjugated with horseradish peroxidase or anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare). Proteins that bound to the antibody were visualized with an ECL kit (GE Healthcare). As reported previously (6), anti-POMT1 and anti-POMT2 polyclonal antibodies did not detect endogenous POMT1 and POMT2, respectively.

Each antibody is specific for the respective recombinant protein; that is, they do not cross-react with each other.

Assay for POMT Activity—POMT activity was based on the amount of mannose transferred from dolichol phosphate mannose to a glutathione *S*-transferase fusion α-DG (GST-αDG) as described previously (6) with a slight modification. Briefly, assays were carried out in a 20-μl reaction volume containing 20 mM Tris-HCl, pH 8.0, 100 nM ^{3}H-labeled dolichol phosphate mannose (Dol-P-[^{3}H]Man, 125,000 dpm/pmol; American Radiolabeled Chemicals, St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg of GST-α-DG, and 80 μg of microsomal membrane fraction. Microsomal fractions were solubilized with buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside for 1 h, and the reaction was initiated by adding Dol-P-[^{3}H]Man.

After a 1-h incubation at 25 °C, the reaction was stopped by adding 150 μl of PBS containing 1% Triton X-100, and the reaction mixture was centrifuged at 10,000 × *g* for 10 min. The supernatant was removed, mixed with 400 μl of PBS containing 1% Triton X-100 and 10 μl of glutathione-Sepharose 4B beads (GE Healthcare), rotated at 4 °C for 1 h, and washed three times with 20 mM Tris-HCl, pH 7.4, containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured with a liquid scintillation counter.

RESULTS

Colocalization of POMT1 and POMT2 in the ER—To determine the subcellular localization of POMT1 and POMT2, HEK293T cells were transfected with expression constructs encoding each protein. Anti-POMT1 and anti-POMT2 antibodies did not stain untransfected HEK293T cells (data not shown), suggesting that HEK293T cells express little POMT1 and POMT2. Myc-tagged POMT1 colocalized precisely with anti-calreticulin (ER marker). Calreticulin staining localized around the nuclei and overlapped with POMT1-Myc staining (Fig. 1*A*). On the other hand, FLAG-tagged POMT2 also colocalized with calreticulin (Fig. 1*B*), in agreement with a previous finding that POMT2 localized to the ER membrane (25). Double staining of HEK293T cells by both POMT1 and POMT2 demonstrated their colocalization (Fig. 1*C*). Thus, we concluded that POMT1 and POMT2 reside in the ER.

Solubilization of POMT Activity—Colocalization of POMT1 and POMT2 in the ER and the requirement of coexpression of both components for protein *O*-mannosylation (6) suggest that POMT1-POMT2 complex formation is necessary for POMT activity. To obtain physical evidence for complex formation between POMT1 and POMT2, we solubilized membrane proteins with various detergents and attempted to detect a complex by immunoprecipitation. In yeast, members of the PMT1 family were found to form heteromeric complexes with members of the PMT2 subfamily *in vivo* by coimmunoprecipitation experiments after solubilization of membrane proteins with 0.35% sodium deoxycholate and 0.5% Triton X-100 (17). Human POMT activity could not be detected when Triton X-100 was used as a detergent, but it could be detected when *n*-octyl-β-D-thioglucoside was used as the detergent (6). Therefore, at first we examined the effect of various detergents on POMT activity of HEK293T cells, in addition to

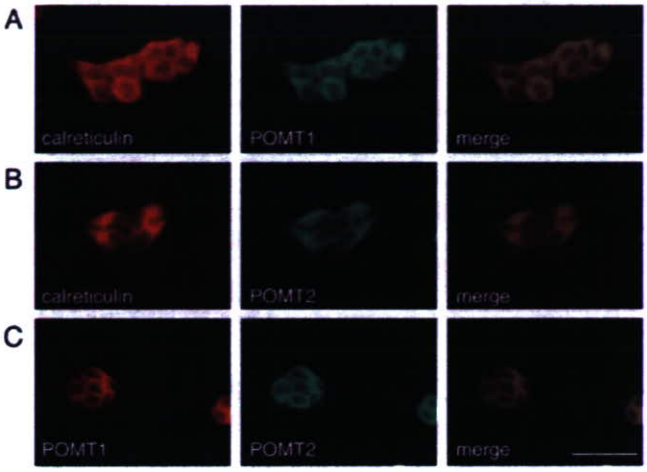

FIGURE 1. **Subcellular localization of POMT1 and POMT2.** *A*, transfected HEK293T cells were stained for calreticulin (*red*) and POMT1-myc (*green*). The merged images show precise co-localization of POMT1 with calreticulin. *B*, transfected HEK293T cells were stained for calreticulin (*red*) and POMT2-FLAG (*green*). The merged images show precise colocalization of POMT2 with calreticulin. *C*, transfected HEK293T cells were stained with fluorescent antibodies against POMT1-Myc (*red*) and POMT2-FLAG (*green*). The merged images show precise colocalization of POMT1 with POMT2. *Scale bar*, 50 μm.

Triton X-100 and *n*-octyl-β-D-thioglucoside. *n*-Octyl-β-D-thioglucoside at a concentration of 0.5% was found to be most effective under our assay conditions (Fig. 2). Then we tried to solubilize POMT activity under various incubation temperatures, incubation times, and detergent concentrations. We found that the optimal conditions for solubilizing POMT activity from the microsomal membrane were 0.5% *n*-octyl-β-D-thioglucoside at 4 °C for 5 h.

Coimmunoprecipitation of POMT1 and POMT2—To determine whether POMT1 and POMT2 form a heterocomplex, POMT1-Myc and POMT2 were cotransfected into HEK293T cells (Fig. 3, *A–C*). Microsomal membrane fractions of these cells were lysed with 0.5% *n*-octyl-β-D-thioglucoside at 4 °C for 5 h and immunoprecipitated with anti-Myc (9E10) antibody-conjugated agarose. A Western blot analysis of precipitates revealed that POMT1-Myc and POMT2 were coimmunoprecipitated (Fig. 3, *D* and *E*, *lanes 1*), indicating that POMT1 and POMT2 form a complex. We then confirmed that POMT2 does not bind to anti-Myc-agarose nonspecifically. Cells that were transfected with only POMT2 were solubilized by *n*-octyl-β-D-thioglucoside and subjected to immunoprecipitation with anti-Myc-agarose. In this case, POMT2 was not detected in the precipitates, indicating POMT2 did not bind to anti-Myc-agarose (Fig. 3, *D* and *E*, *lanes 3*). On the other hand, when POMT1-Myc and POMT2 were separately expressed in different cells and then solubilized, mixed, and immunoprecipitated with anti-Myc-agarose, no complex formation between POMT1-Myc and POMT2 was detected (Fig. 3, *D* and *E*, *lanes 5*). These results indicated that the POMT1 and POMT2 proteins could not associate with each other when they are expressed separately and suggest that assembly of POMT1 and POMT2 occurs in the ER membrane.

Next, we examined whether the coprecipitated POMT1-POMT2 complex has POMT activity. Distinct activity was detected in the precipitates from POMT1-Myc-POMT2

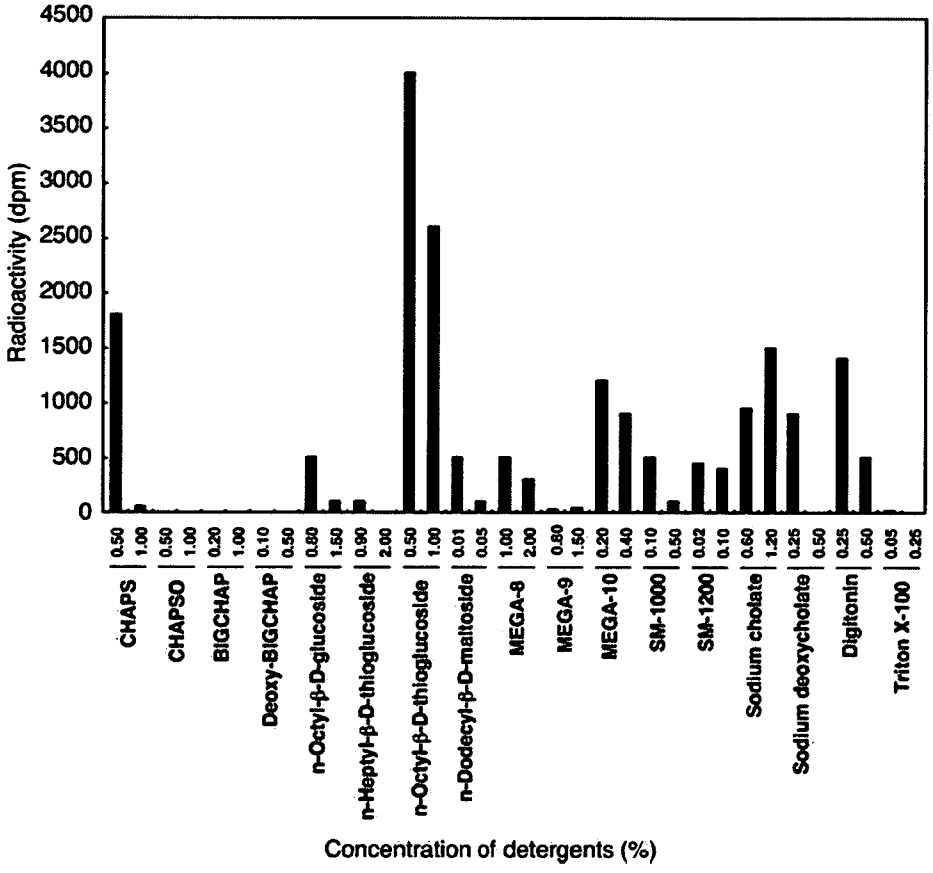

FIGURE 2. **Effect of detergents on POMT activity.** *CHAPS*, 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid; *CHAPSO*, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid; *BIGCHAP*, *N,N*-bis(3-D-gluconamidopropyl) cholamide; *Deoxy-BIGCHAP*, *N,N*-bis(3-D- gluconamidopropyl)deoxycholamide; *MEGA-8*, *n*-octanoyl-*N*-methylglucamide; *MEGA-9*, *n*-nonanoyl-*N*-methylglucamide; *MEGA-10*, *n*-decanoyl-*N*-methylglucamide; *SM-1000*, β-D-fructopyranosyl-α-D-glucopyranosidemonodecanoate; *SM-1200*, β-D-fructopyranosyl-α-D-glucopyranoside monododecanoate.

cotransfected cells (Fig. 3*F*, *lane 1*). The precipitates from cell membranes expressing only POMT1-Myc had slight POMT activity (Fig. 3*F*, *lane 2*). This weak activity may be due to complex formation between transfected POMT1-Myc and endogenous POMT2. The POMT activity in a mixture of individually expressed POMT1-Myc and POMT2 was similar to the background level (Fig. 3*F*, *lane 5*). Based on these results, we concluded that POMT1 and POMT2 associate physically *in vivo* and that this state becomes functional.

Effect of Mutations on POMT Activity—Recently, three mutations (G65R, A200P, and W582C) in the *POMT1* gene were found (Table 1) (11–13). Because these patients have milder phenotypes than typical WWS patients, we expected the mutated POMT1s to have some POMT activity. To test this hypothesis, these mutations were introduced into *POMT1-myc* cDNA and cotransfected into HEK293T cells with *POMT2* (Fig. 4, *A–C*). However, none of the POMT1 mutants, like the other mutants (10), showed any POMT activity (Fig. 4*D*).

Immunoprecipitation of Mutant POMT1 and POMT2— Eight mutations in the *POMT1* gene of patients with WWS (G76R, L421del, V428D, G65R, A200P, W582C, V703fs, and G722fs) were found to abolish POMT activity in both the present and previous studies (10). We examined six of these mutant POMT1s (all but the latter two) to determine whether they prevented complex formation with POMT2 (Table 1). The mutated *POMT1-mycs* were cotransfected into HEK293T cells with *POMT2*. POMT2 was found to precipitate with each of the POMT1 mutants (Fig. 5, *A* and *B*), indicating that these mutations did not affect complex formation with POMT2.

DISCUSSION

Protein *O*-mannosylation is an essential post-translational modification (19). In yeast and fungi, protein *O*-mannosylation is indispensable for cell wall integrity and normal cellular morphogenesis (16). In *Drosophila*, a defect of *O*-mannosylation causes a rotation of the abdomen due to abnormal muscle development (26, 27). In mouse, targeted disruption of the *Pomt1* gene is embryonically lethal (28). In human, impairment of α-DG *O*-mannosylation leads to congenital muscular dystrophy and neuronal migration disorders, WWS (7, 10). Protein *O*-mannosylation requires at least two components, POMT1 and POMT2 (6). The present results have shown that POMT1 and POMT2 form a heterocomplex *in vivo*. The immunoprecipitated complex of POMT1 and POMT2 possessed POMT activity. In addition, we demonstrated that mutant POMT1s that have been found in WWS patients have the ability to form a complex with POMT2, although they lost POMT activities.

In yeast, the members of the PMT1 subfamily interact heterophilically with those of the PMT2 subfamily, whereas the single member of the PMT4 subfamily acts as a homophilic complex (17, 24). On the other hand, in human the single member of the PMT2 subfamily (POMT2) interacts with a member of the PMT4 subfamily (POMT1). These results suggest that the combination of interacting molecules has changed during evolution. It is noteworthy that only a single PMT member has been found in *M. tuberculosis* and that it has POMT activity (18). It is unclear whether it forms a homophilic complex *in vivo*.

Yeast PMTs and human POMTs are predicted to be integral membrane proteins with multiple transmembrane domains (25, 29). However, human and yeast POMT proteins showed differences in detergent sensitivity. Triton X-100 appeared to abolish human POMT activity (6) but did not inhibit yeast PMT activity (30). This difference may be due to the lipid compositions in human and yeast, which would affect the efficiency of

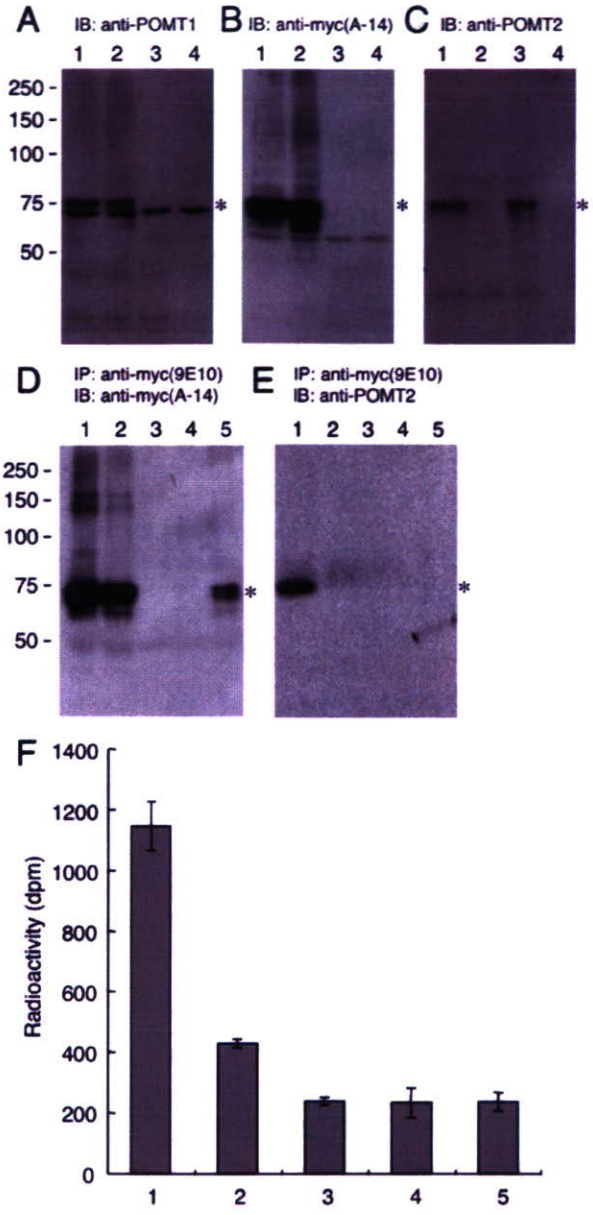

FIGURE 3. **Physical and functional association of POMT1 and POMT2.** *A–C*, expression of POMT-Myc and POMT2 in HEK293T microsomal membrane fractions was determined by anti-POMT1 antibody (*A*), anti-Myc antibody (A-14) (*B*), and anti-POMT2 antibody (*C*). *D* and *E*, POMT1-POMT2 complex formation *in vivo*. POMT1-Myc and POMT2 were transfected into HEK293T cells and immunoprecipitated by anti-Myc (9E10) antibody-conjugated agarose. The resulting precipitates were analyzed by immunoblotting with anti-Myc antibody (A-14) (*D*) and anti-POMT2 antibody (*E*). *F*, POMT activity of immunoprecipitates. *Lane 1*, POMT1-Myc and POMT2 were transfected into cells; *lane 2*, cells were transfected with POMT1-Myc alone; *lane 3*, cells were transfected with POMT2 alone; *lane 4*, mock transfectant; *lane 5*, a mixture of individually expressed POMT1-Myc and POMT2. *Asterisks* indicate positions of corresponding molecules. Molecular weight standards are shown on the *left*. POMT activity was based on the amount of mannose transferred to a GST-αDG. Average values of three independent experiments are shown.

protein solubilization by detergents and the stability of proteins after the removal of lipids by detergents.

Yeast Pmt1p has been proposed to consist of seven transmembrane helices (31). The Pmt1p N terminus and loops 2, 4, and 6 are located in the cytoplasm, and the C terminus and

TABLE 1
Summary of mutations in the *POMT1* gene of WWS patients

Mutations	Effects
G193A	Gly^{65} → Arg missense (G65R)
G598C	Ala^{200} → Pro missense (A200P)
G1746C	Trp^{582} → Cys missense (W582C)
C226A	Gly^{76} → Arg missense (G76R)
1260 to 1262 del CCT	Leu^{421} deletion (L421del)
T1283A	Val^{428} → Asp missense (V428D)

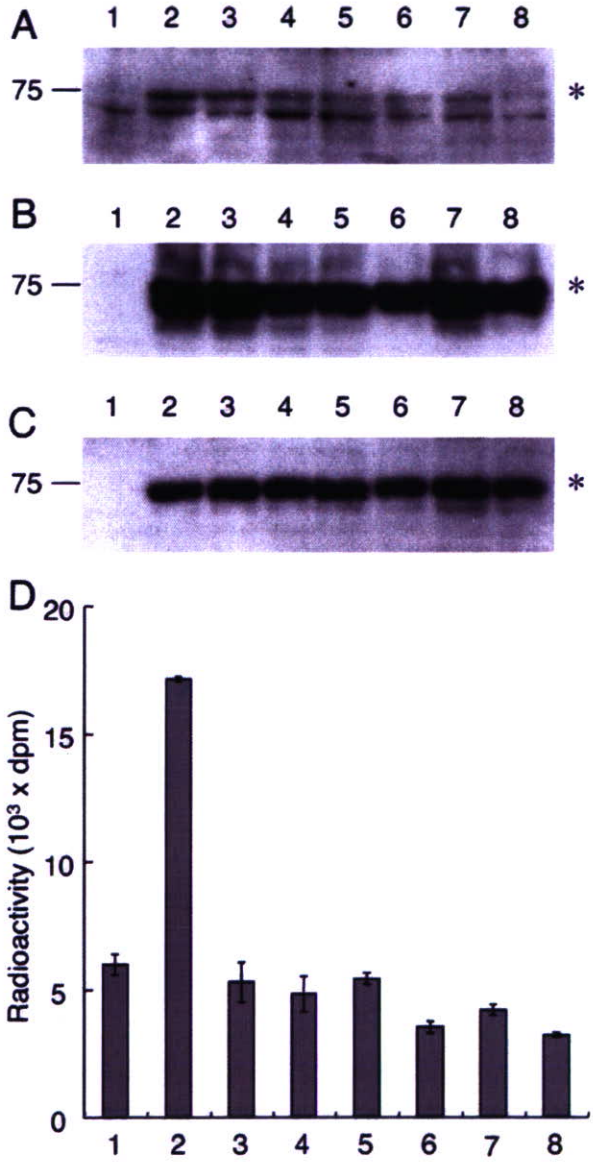

FIGURE 4. **Enzymatic activity mutated POMT1-Myc with POMT2.** *A–C*, Western blot analyses of POMT1-Myc and POMT2 proteins detected by anti-POMT1 antibody (*A*), anti-Myc antibody (A-14) (*B*), and anti-POMT2 antibody (*C*). *D*, POMT activities of the POMT1-Myc mutants coexpressed with POMT2. *Lane 1*, mock; *lane 2*, POMT1-Myc and POMT2; *lane 3*, G65R and POMT2; *lane 4*, A200P and POMT2; *lane 5*, W582C and POMT2; *lane 6*, G76R and POMT2; *lane 7*, L421del and POMT2; *lane 8*, V428D and POMT2. *Asterisks* indicate the migration positions of each POMT1-Myc protein (*A*, *B*) and POMT2 protein (*C*). Molecular weight standards are shown on the *left*. POMT activity was based on the amount of mannose transferred to a GST-αDG. Average values of three independent experiments are shown.

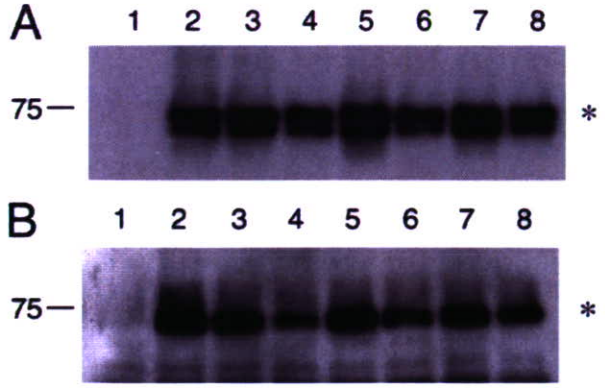

FIGURE 5. **Complex formation of mutated POMT1-Myc with POMT2.** *A* and *B*, immunoprecipitates of mutated POMT1-Myc and POMT2 proteins analyzed with anti-Myc antibody (A-14) (*A*) and anti-POMT2 antibody (*B*). *Lanes 1*, mock; *lanes 2*, POMT1-Myc and POMT2; *lanes 3*, G65R and POMT2; *lanes 4*, A200P and POMT2; *lanes 5*, W582C and POMT2; *lanes 6*, G76R and POMT2; *lanes 7*, L421del and POMT2; *lanes 8*, V428D and POMT2. *Asterisks* indicate the migration positions of each POMT1-Myc protein (*A*) and POMT2 protein (*B*). Molecular weight standards are shown on the *left*.

loops 1, 3, and 5 are located in the ER lumen. A large hydrophilic region (loop 5) and loop 1 are important for enzymatic activity (17, 31, 32). Based on this model, seven of ten *POMT1* mutations identified in WWS patients appear to be located in loops 1 and 5 (7, 9, 11–13), and three of four *POMT2* mutations identified in WWS patients are located in loop 5 (8). Taken together, these results indicate that loops 1 and 5 are important for catalysis, as they are in yeast PMTs. Furthermore, deletion of loop 5 in yeast Pmt1 eliminates enzymatic activity, but not Pmt1-Pmt2 interactions (32). Similarly, mutant POMT1 has the ability to form a complex with POMT2, although the complex does not have POMT activity. Further studies are needed to clarify the role of each domain in POMT activity and complex formation. Additionally, it has been shown that an Arg residue in the transmembrane domain is necessary for complex formation in yeast PMTs and a Glu residue in loop 1 is necessary for enzymatic activity (32). These amino acid residues are conserved in human POMT1 (33), and so it will be interesting to see whether they also are necessary for complex formation and activity. It is also of interest to screen uncharacterized WWS patients for the Arg and Glu mutations.

There is growing evidence that the glycans of glycoproteins play several roles in cellular differentiation and developmental events as well as in disease processes (34). Glycosylation is basically controlled by the combined action of many glycosyltransferases. The level and the strict substrate specificity of glycosyltransferases cooperate to synthesize specific sugar sequences and sugar linkages found in glycoproteins. Glycosyltransferase activities are regulated by other factors or by complex formation. For example, human core 1 β3-galactosyltransferase activity requires the expression of Cosmc (35). Cosmc is a molecular chaperone that specifically assists the folding/stability of core 1 β3-galactosyltransferase and is required for a glycosyltransferase expression. Mutations of *COSMC* were recently found in patients with Tn syndrome who could not produce core 1 structure (Galβ1–3GalNAc) (36). Another glycosyltransferase with complex regulation is human chondroitin synthase, which cannot polymerize chondroitin sulfate *in vitro*; rather, its activity requires the coexpression of chondroitin-polymerizing factor (37). As a third example, the bifunctional glycosyltransferases EXT1 and EXT2, which polymerize heparan sulfate, need to form a hetero-oligomeric complex to exert their optimal catalytic activities and to exist in the appropriate intracellular locations (38, 39). In the present study, we observed that protein *O*-mannosylation can be initiated by direct complex formation of POMT1 and POMT2, but not by either enzyme by itself. POMT1 or POMT2 are thus different from EXT1 and EXT2 because the latter enzymes are active by themselves. One possibility is that formation of the POMT1-POMT2 complex creates a new catalytic domain. Further studies are needed to elucidate the mechanism of complex formation between POMT1 and POMT2 and the regulation of POMT activity. Our results, together with previous studies of glycosyltransferases, indicate that glycosylation is regulated in a complicated fashion.

Our findings that POMT1 and POMT2 associated physically and functionally *in vivo* and that POMT1 and POMT2 could not associate when they are expressed individually and then mixed suggest that the assembly of POMT1 and POMT2 requires specific conditions in the ER membrane. However, a heterocomplex POMT2 and mutated POMT1s suggest that single amino acid substitutions and deletion in POMT1 found in WWS patients do not affect assembly of POMT1 and POMT2. These mutations would abolish dolichol phosphate mannose or acceptor (α-DG) binding. In the POMT assay, cells transfected with mutated POMT1 and wild-type POMT2 (Fig. 4*D*, *lanes 3–8*) had decreased enzymatic activity compared with the mock transfectant (Fig. 4*D*, *lane 1*). The decrease of endogenous enzymatic activity may be caused by the disturbance of endogenous POMT1-POMT2 formation.

WWS patients carrying three mutations (G65R, A200P, and M582C) showed milder phenotypes than typical WWS (14, 15), which led us to expect that these mutations would not completely abolish activity. Our finding that these mutant proteins did not have any enzymatic activity is thus puzzling. One possibility is that the levels of LARGE expression may be greater in these patients than in previous reported severe WWS patients. Barresi *et al.* (40) report that overproduction of LARGE caused hyperglycosylation of α-DG and improvement of function, *e.g.* laminin binding, in WWS fibroblasts. However, the change of the LARGE expression level in WWS patients may be rare because these patients had hypoglycosylated α-DG (14, 15). Measurement of POMT activities of tissues from these patients would help to explain their mild symptoms. If each patient showed some POMT activities, other factor(s) that regulate POMT activity should be considered. In fact, some WWS patients have no mutations in *POMT1* or *POMT2* (7, 11). It is possible that mutations of such factors may cause unidentified WWS. Further studies are needed to test this hypothesis.

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Molecular cloning and characterization of rat Pomt1 and Pomt2

Hiroshi Manya[2]*, Atsuro Chiba[3]*, Richard U. Margolis[4], and Tamao Endo[1,2]

[2]Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015, Japan; [3]Department of Neurology, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan; and [4]Department of Pharmacology, New York University Medical Center, New York, NY 10016

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Mammalian *O*-mannosylation, although an uncommon type of protein modification, is essential for normal brain and muscle development. Defective *O*-mannosylation causes congenital muscular dystrophy with abnormal neuronal migration [Walker–Warburg syndrome (WWS)]. Here, we have identified and cloned rat *Pomt1* and *Pomt2*, which are homologues of human *POMT1* and *POMT2*, with identities of 86 and 90%, respectively, at the amino acid level. Coexpression of both genes was found to be necessary for enzymatic activity, as is the case with human *POMT1* and *POMT2*. Northern blot and reverse transcriptase polymerase chain reaction (RT–PCR) analyses revealed that rat *Pomt1* and *Pomt2* are expressed in all tissues but most strongly in testis. *In situ* hybridization histochemistry of rat brain revealed that *Pomt1* and *Pomt2* mRNA are coexpressed in neurons (dentate gyrus and CA1-CA3 region of the hippocampus and cerebellar Purkinje cells). Two transcription-initiation sites were observed in rat *Pomt2*, resulting in two forms: a testis form and a somatic form. The two forms had equal protein *O*-mannosyltransferase activity when coexpressed with rat Pomt1. Coexpression studies also showed that the human and rat protein *O*-mannosyltransferases are interchangeable, providing further evidence for the closeness of their structures.

Key words: glycosylation/Pomt1 and Pomt2/protein *O*-mannosyltransferase activity/rat

Introduction

Mammalian *O*-mannosylation is an uncommon type of protein modification that was first identified in chondroitin sulfate proteoglycans of brain and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle (Finne *et al.*, 1979; Krusius *et al.*, 1986, 1987; Endo, 1999). α-Dystroglycan (α-DG) is an *O*-mannosyl-modified glycoprotein that is a central component of the dystrophin–glycoprotein complex isolated from skeletal muscle membranes (Michele and Campbell, 2003). We previously found that the glycans of α-DG include *O*-mannosyl oligosaccharides and that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is a laminin-binding ligand of α-DG (Chiba *et al.*, 1997). Subsequently, a series of *O*-mannosyl glycans with different mannose branching and peripheral structures were found in mammals (Yuen *et al.*, 1997; Sasaki *et al.*, 1998; Smalheiser *et al.*, 1998; Endo, 1999).

Initiation of protein *O*-mannosylation has been partially characterized. In yeast, a family of protein *O*-mannosyltransferases (*pmt1~7*) catalyzes the transfer of a mannosyl residue from dolichyl phosphate mannose (Dol-P-Man) to Ser/Thr residues of certain proteins (Strahl-Bolsinger *et al.*, 1999). In humans, two homologues, *POMT1* and *POMT2*, are present (Jurado *et al.*, 1999; Willer *et al.*, 2002, 2004). Human POMT1 and POMT2 share almost identical hydropathy profiles that predict both to be integral membrane proteins with multiple transmembrane domains. Recently, we demonstrated that human POMT1 and POMT2 have protein *O*-mannosyltransferase activity, but only when they are coexpressed, and later we found that human POMT1 and POMT2 form a heterocomplex to express enzymatic activity (Manya *et al.*, 2004). This has also been found to be the case in *Drosophila*. Two orthologs of human *POMT* genes, *dPOMT1* and *dPOMT2*, are present, and both are required for protein *O*-mannosylation (Ichimiya *et al.*, 2004).

Protein *O*-mannosylation is important for normal brain and muscle development, because a defect of *O*-mannosylation causes congenital muscular dystrophy with abnormal neuronal migration (Endo, 2004), the so-called Walker–Warburg syndrome (WWS: OMIM 236670) (Dobyns *et al.*, 1989). Patients with WWS are severely affected from birth and usually die within their first year. Recently, WWS patients have been found to have mutations in both *POMT1* and *POMT2* (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). In WWS patients, a highly glycosylated α-DG was selectively deficient in skeletal muscle (Beltran-Valero de Bernabe *et al.*, 2002; Jimenez-Mallebrera *et al.*, 2003; van Reeuwijk *et al.*, 2005). This finding suggests that α-DG is a potential target of POMT1 and POMT2 and that hypoglycosylation of α-DG may be a pathomechanism of WWS. In fact, *POMT1* mutations found in WWS patients led to a defect of protein *O*-mannosyltransferase activity even when the defective *POMT1* was coexpressed with wild-type *POMT2* (Akasaka-Manya *et al.*, 2004). In *Drosophila*, functional dPOMT1 and dPOMT2 are required for normal muscle development (Ichimiya *et al.*, 2004).

In this study, we isolated rat orthologs of *POMT1* and *POMT2* cDNA clones and determined in which tissues they

[1]To whom correspondence should be addressed; e-mail: endo@tmig.or.jp
*These contributed equally to this work.

The nucleotide sequences reported in this article have been submitted to the DDBJ/GenBank/EBI Data Bank with accession numbers AF192388 for rat Pomt1 and AB246667 for rat Pomt2.

are expressed. We also examined the distribution of protein *O*-mannosyltransferase activity in various rat tissues.

Results

cDNA cloning of rat Pomt1 and Pomt2

A rat *Pomt1* cDNA was obtained by reverse transcriptase polymerase chain reaction (RT–PCR) using a rat brain cDNA library. The nucleotide sequence of the cDNA is predicted to encode a protein of 748 amino acids (Figure 1A). In addition, two rat *Pomt2* cDNAs were obtained by RT–PCR using a rat testis cDNA library. These two cDNAs originated from alternative initiation sites. The longer and shorter forms are predicted to contain proteins of 810 and 740 amino acids, respectively (Figure 1B). The former is named *t-Pomt2* and the latter is *s-Pomt2* (somatic form), because *t-Pomt2* expression is highly specific to the testis as described below.

ClustalW alignments show that human, mouse, and rat POMT1s (Figure 2A) are closely related and that human, mouse, and rat POMT2s (Figure 2B) are closely related. Rat Pomt1 and Pomt2 showed 86 and 90% identities to human POMT1 and POMT2, and 96 and 97% identities to mouse Pomt1 and Pomt2, respectively.

Expression of rat Pomt1 and Pomt2 genes

To examine the expression patterns and the size of rat *Pomt1* and *Pomt2* mRNAs, northern blot analyses were performed (Figure 3A). The mRNA band of around 3.3 kb represents the basic transcript of *Pomt1*. *Pomt1* mRNA was expressed in all tissues and predominantly expressed in testis. The basic transcript of *Pomt2* was around 2.7 kb, but due to alternative polyadenylation, 3.7 and 4.7 kb mRNAs were also detected (closed triangles in Figure 3A, middle panel). In testis, the transcript sizes were slightly larger due to differential transcription initiation (open triangles in Figure 3A, middle panel). Like *Pomt1*, *Pomt2* was expressed in all tissues but predominantly in testis.

The more sensitive RT–PCR analyses of rat *Pomt1*, *Pomt2*, and *t-Pomt2* were performed (Figure 3B). PCR products of *Pomt1* and *Pomt2* were detected in all tissues (top and second panels in Figure 3B). However, *t-Pomt2* mRNA was predominantly expressed in testis and slightly detected in brain, lung, and liver (third panel of Figure 3B). Differential transcription initiation of *Pomt2* gene was observed in mouse (Willer *et al.*, 2002), and the longer transcript is restricted to testis.

As shown by *in situ* hybridization, the messages of *Pomt1* and *Pomt2* were coexpressed in rat brain hippocampus and cerebellar cortex (Figure 4). Both mRNAs were mainly expressed in the cells of gray matter and strongly expressed in neurons of the dentate gyrus and CA1-CA3 region in the hippocampus formation and in Purkinje cells in the cerebellar cortex.

Protein O-mannosyltransferase activities in rat tissues

High protein *O*-mannosyltransferase activities were observed in brain, kidney, and testis (Figure 5). The activity in spleen was low, in agreement with the low levels of expression of *Pomt1* and *Pomt2* in spleen (Figure 3A).

Protein O-mannosyltransferase activity of the cloned cDNA products

To analyze the protein *O*-mannosyltransferase activity of rat Pomt1 and Pomt2, the expression vector of the cloned cDNAs was transfected into HEK293T cells, and the microsomal membranes were used for enzymatic assay as described under *Materials and Methods*. Expressed proteins were shown by staining with anti-POMT1 antibody (Figure 6A) and anti-POMT2 antibody (Figure 6B). Protein *O*-mannosyltransferase activity was observed when rat Pomt1 and s-Pomt2 were coexpressed (Figure 6C, lane 6), but not when they were expressed independently (Figure 6C, lanes 2 and 3). α-Mannosidase digestion showed that the mannosyl residue was linked to α-DG by α-linkage (data not shown), as reported previously (Manya *et al.*, 2004).

Cells cotransfected with rat *Pomt1* and human *POMT2* (Figure 6C, lane 8) and cells cotransfected with human *POMT1* and rat *s-Pomt2* (Figure 6C, lane 9) showed comparative protein *O*-mannosyltransferase activities with cells cotransfected rat *Pomt1* and rat *s-Pomt2* (Figure 6C, lane 6) and cells cotransfected human *POMT1* and human *POMT2* (Figure 6C, lane 7). As expected, cells cotransfected rat *Pomt1* and human *POMT1* and cells cotransfected rat *s-Pomt2* and human *POMT2* did not show enzymatic activity (data not shown).

Protein O-mannosyltransferase activities of rat s-Pomt2 and t-Pomt2

Rat *s-Pomt2* and *t-Pomt2* were expressed with or without rat *Pomt1* in HEK293T cells as shown by staining with anti-POMT1 antibody (Figure 7A) and anti-POMT2 antibody (Figure 7B). Cells cotransfected with rat *Pomt1* and *s-Pomt2* (Figure 7, lane 3) and cells cotransfected with rat *Pomt1* and *t-Pomt2* (Figure 7, lane 4) had comparable protein *O*-mannosyltransferase activities, but cells expressing only t-Pomt2 or s-Pomt2 had little activity (Figure 7C, lanes 1 and 2). These results demonstrate that coexpression of t-Pomt2 or s-Pomt2 with rat Pomt1 showed similar enzymatic activities.

Discussion

In this study, we identified and cloned rat *Pomt1* and *Pomt2*. We also proved that protein *O*-mannosyltransferase activity is encoded in both genes, because coexpression of both genes was necessary for the enzymatic activity. Northern blot and RT–PCR analyses revealed that rat *Pomt1* and *Pomt2* are expressed strongly in the testis and weakly in all other tissues examined. However, enzyme activity is almost the same in brain, kidney, and testis. This may be due to the fact that mRNA levels do not always correlate well with actual protein expression. We do not know the expression levels of Pomt proteins in each tissue because we do not have antibodies that recognize the endogenous Pomts. Another possibility is variations in the presence or absence of activators, cofactors, or inhibitors of protein *O*-mannosyltransferase activity in the different tissues. In fact, some WWS patients have no mutations in *POMT1* or *POMT2* (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). It is

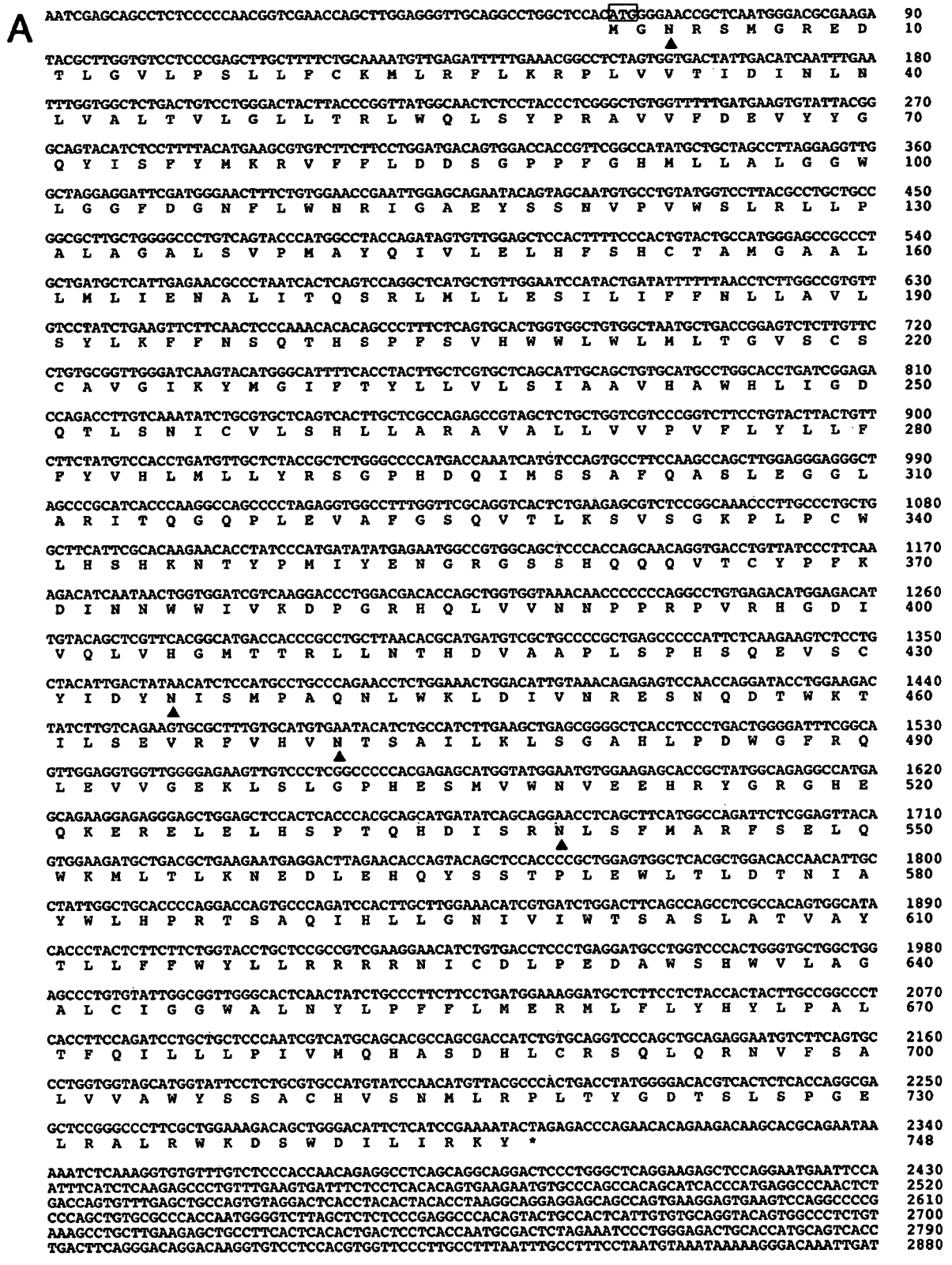

Fig. 1. Nucleotide and deduced amino acid sequences of rat *Pomt1* (**A**) and *Pomt2* (**B**). The cDNA sequences of rat *Pomt1* and *Pomt2* are listed in the top line. Deduced amino acid sequences are indicated by the single-letter amino acid codes. Potential *N*-glycosylation sites are indicated by filled triangles. ATG-start codons are boxed. In *Pomt2* (**B**), the *N*-terminal extension of the deduced testis-specific isoform is underscored and the testis-specific 5′-sequence is shaded.

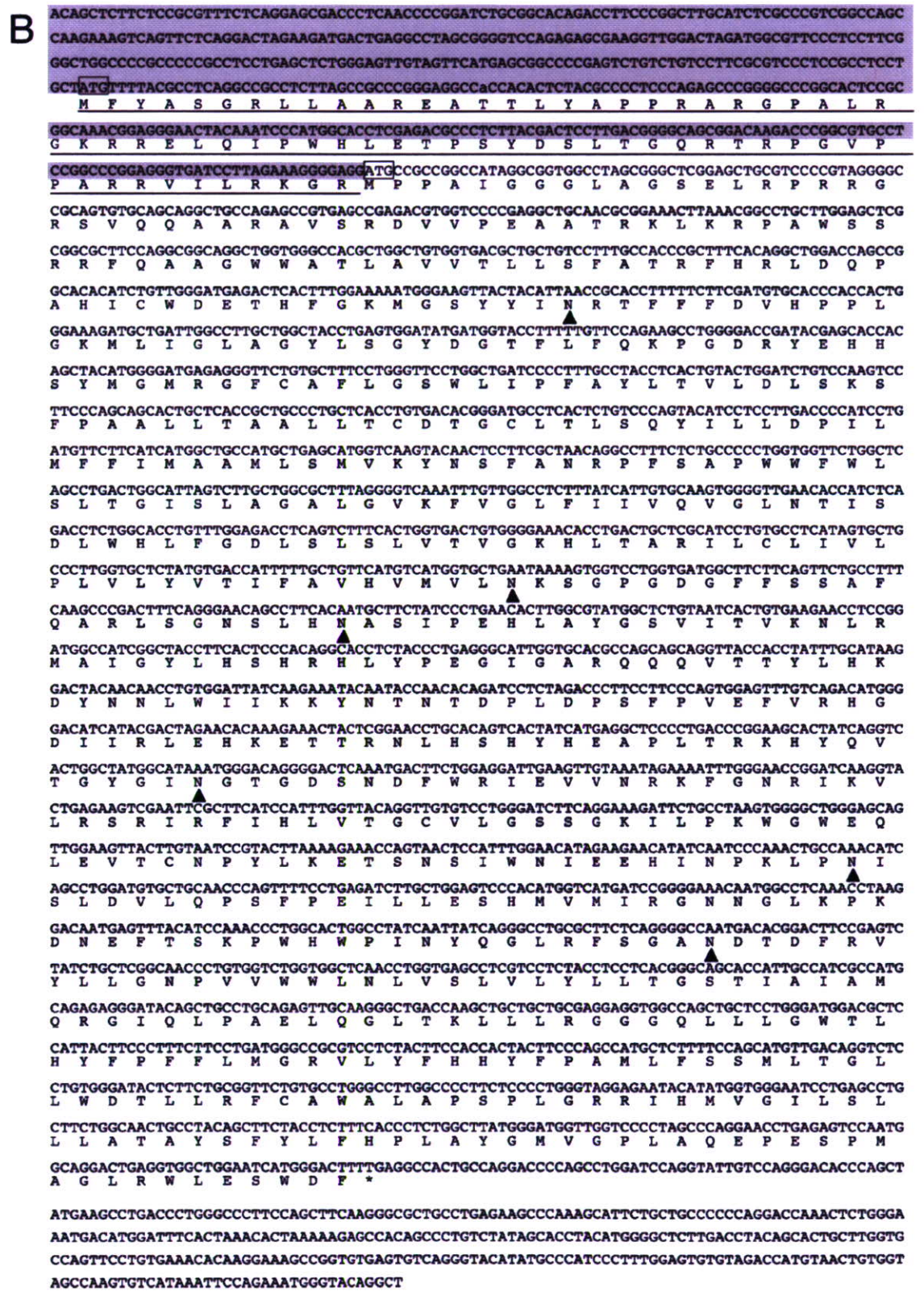

Fig. 1. continued

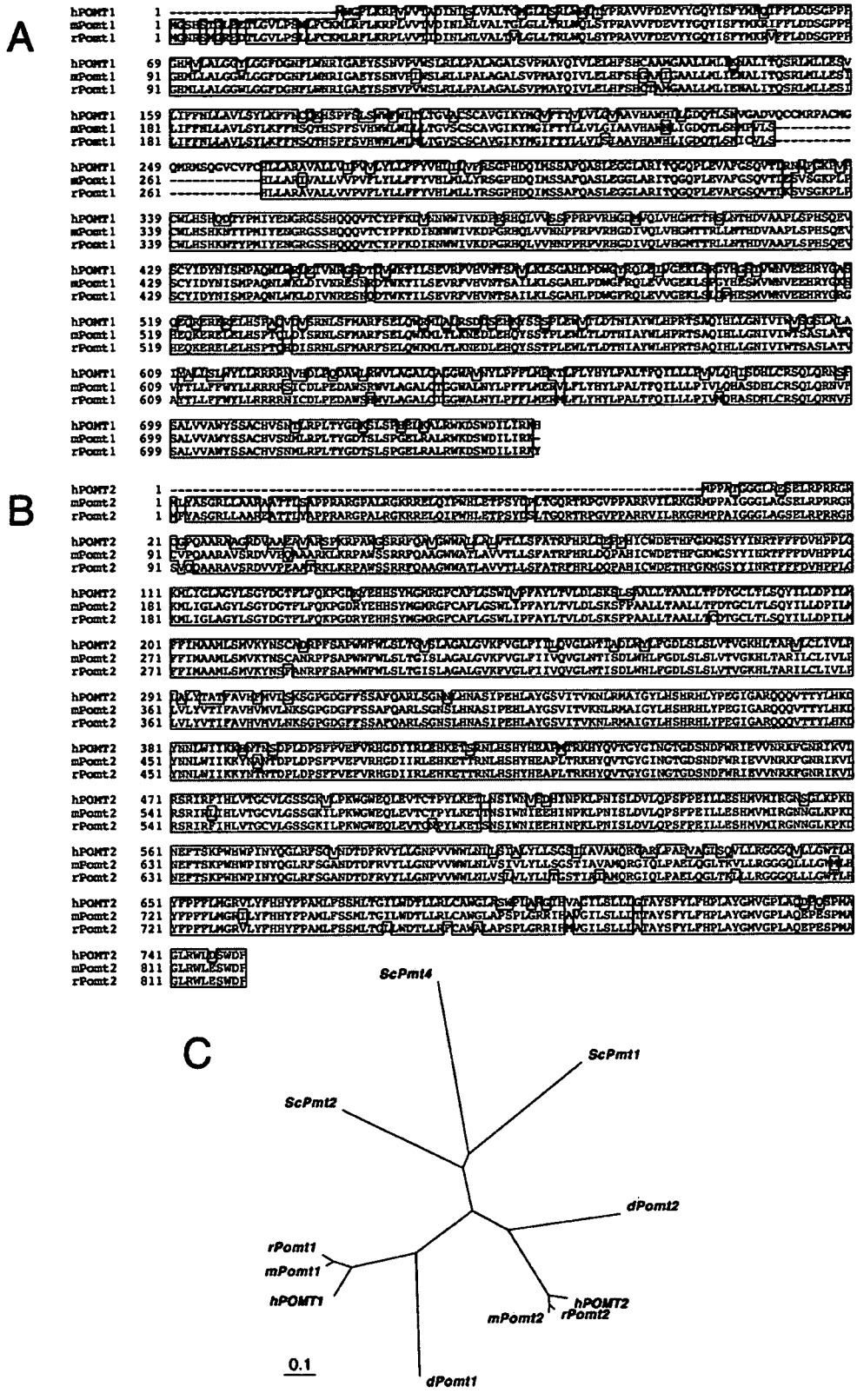

Fig. 2. Comparison of human, mouse, and rat POMTs. (**A** and **B**) ClustalW alignments of human, mouse and rat POMT1 and POMT2 amino acid sequences, respectively. Conserved amino acids are boxed. (C) ClustalW phylogenic tree of human, mouse, rat, and *Drosophila* POMTs and *Saccharomyces cerevisiae* Pmt1, Pmt2, and Pmt4. The branch lengths indicate amino acid substitutions per site. h, human; m, mouse; r, rat.

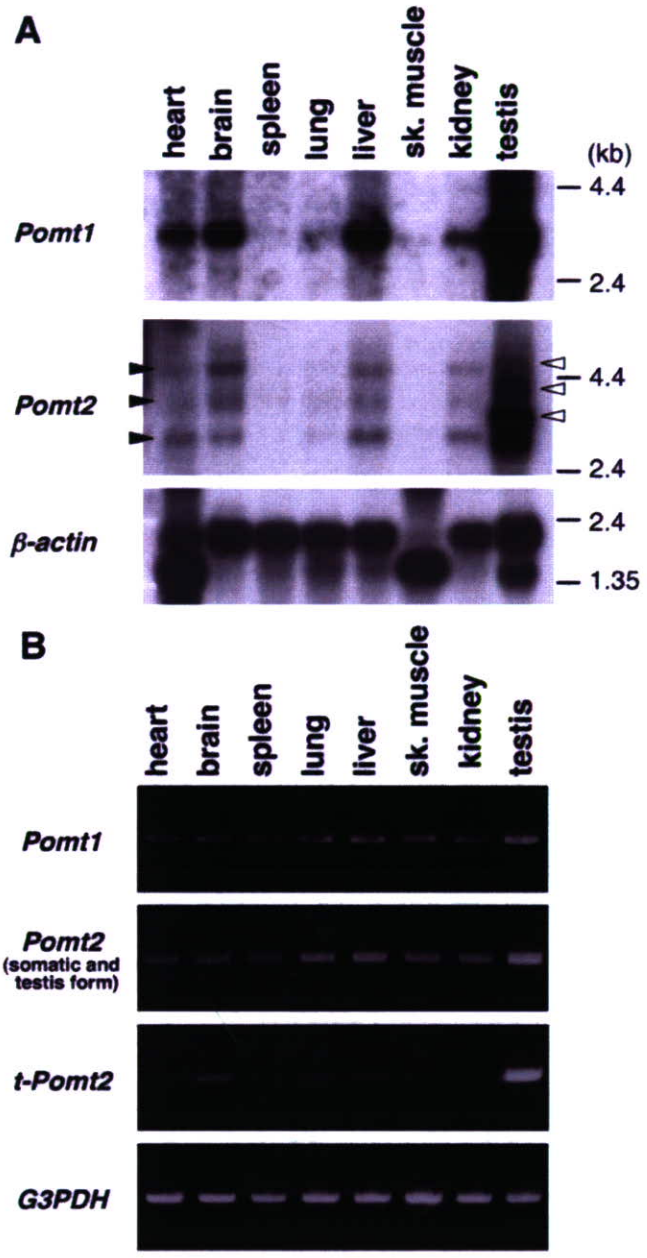

Fig. 3. Expression of rat *Pomt1* and *Pomt2* genes. (**A**) Northern blot analysis probed with rat *Pomt1* (upper panel), rat *Pomt2* (middle panel) and β-actin (lower panel). Each lane contains 2 μg poly(A)$^+$ RNA (Rat MTN blot). The sizes of RNA marker bands are indicated on the right. The basic transcript of *Pomt2* was around 2.7 kb, but because of alternative polyadenylation, 3.7 kb and 4.7 kb mRNAs of *Pomt2* were also detectable (closed triangles). In testis, the transcript sizes were slightly larger due to differential transcription initiation (open triangles). (**B**) RT–PCR analysis of rat *Pomt1* (top panel), rat *Pomt2* (second panel), testis form of rat *Pomt2* (third panel), and rat *G3PDH* (bottom panel) mRNAs. PCR products of *Pomt1* and *Pomt2* were detected in all tissues. Testis form of *Pomt2* mRNA was predominantly expressed in testis and slightly detected in brain, lung, and liver. sk. muscle, skeletal muscle.

therefore possible that mutations of other factors that regulate POMT activity may cause unidentified WWS, although further studies are needed to test this hypothesis.

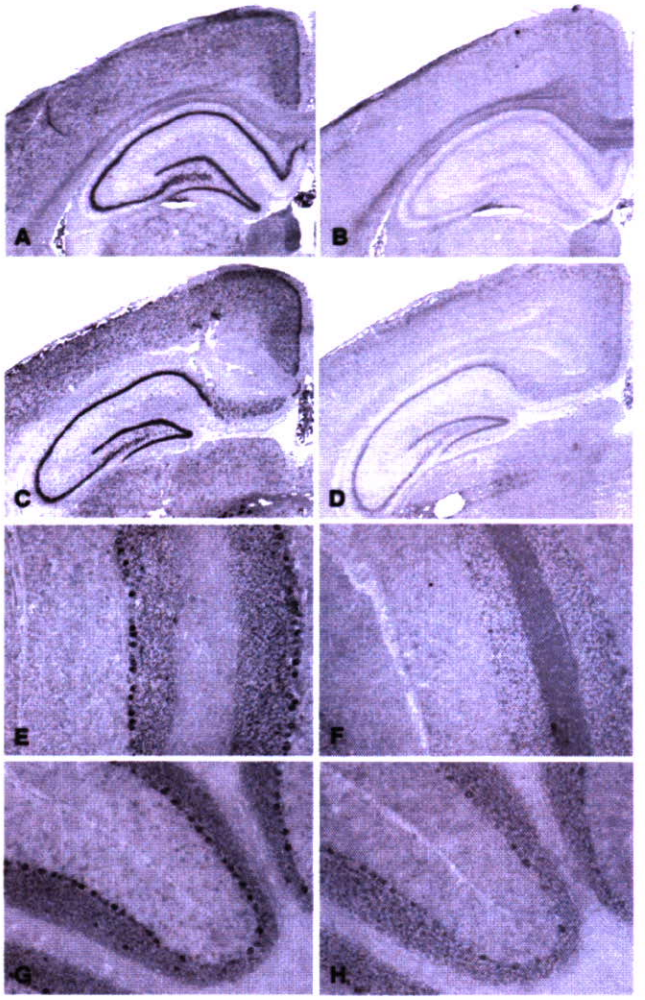

Fig. 4. Colocalization of rat *Pomt1* and *Pomt2* mRNA in adult rat brain. *In situ* hybridization histochemistry of adult rat cerebrum (**A–D**), and cerebellar cortex (**E–H**) shows the same mRNA expression pattern of rat *Pomt1* (**A** and **E**, antisense probe; **B** and **F**, sense probe) and *Pomt2* (**C** and **G**, antisense probe; **D** and **H**, sense probe). Both of the mRNAs are mainly expressed in cells in gray matter and strongly expressed in neurons of the dentate gyrus and CA1-CA3 region in hippocampus formation and Purkinje cells in cerebellar cortex.

Pomt2 was found to have two transcription-initiation sites, giving rise to the testis form (*t-Pomt2*) and the somatic form (*s-Pomt2*). Both t-Pomt2 and s-Pomt2 showed protein *O*-mannosyltransferase activity equally when coexpressed with rat Pomt1. In addition, human POMT1 and POMT2 could be exchanged for rat Pomt1 and Pomt2, without loss of activity. Human POMT1 and rat Pomt1 proteins share 96% similarity and 86% identity; human POMT2 and rat Pomt2 share 99% similarity and 90% identity. Such high similarity may explain the exchangeability of each component for enzymatic activity. The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weights of t-Pomt2 and s-Pomt2 expressed in HEK293T cells were 85 and 75 kDa, respectively. The difference was due to the presence of an additional 70 amino acids in the *N*-terminal region of t-Pomt2. However, this difference did

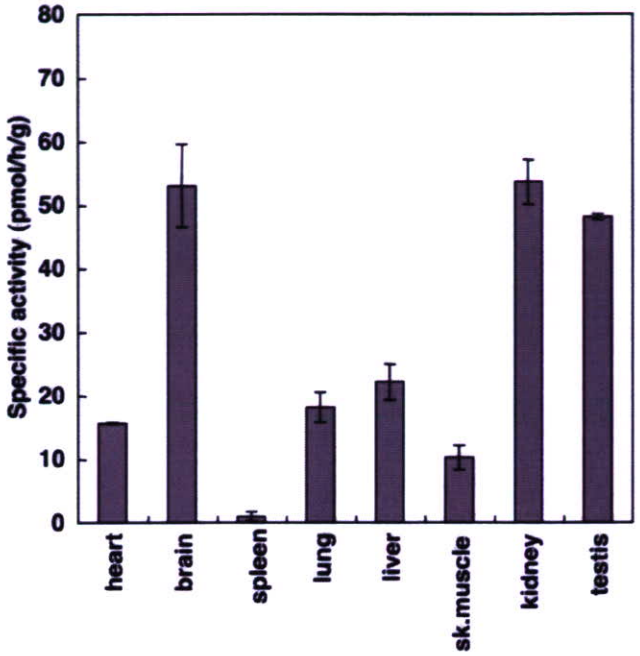

Fig. 5. Protein *O*-mannosyltransferase activity in rat tissues. Protein *O*-mannosyltransferase activity was measured in 0.5 mL Eppendorf tubes in a 20 μL reaction volume containing 20 mM Tris–HCl (pH 8.0), 100 nM of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol), 0.5% *n*-octyl-β-D-thioglucoside, 2 mM 2-mercaptethanol, 10 mM EDTA, 10 μg GST-αDG, and 80 μg of microsomal membrane fraction. The reaction was initiated by adding the protein extract and continued at 25°C for 1 h. After incubation, GST-αDG was separated by Glutathione-Sepharose 4B beads and then the incorporated [^{3}H]-mannose to GST-αDG was measured with a liquid scintillation counter. Average values of three independent experiments are shown. sk. muscle, skeletal muscle.

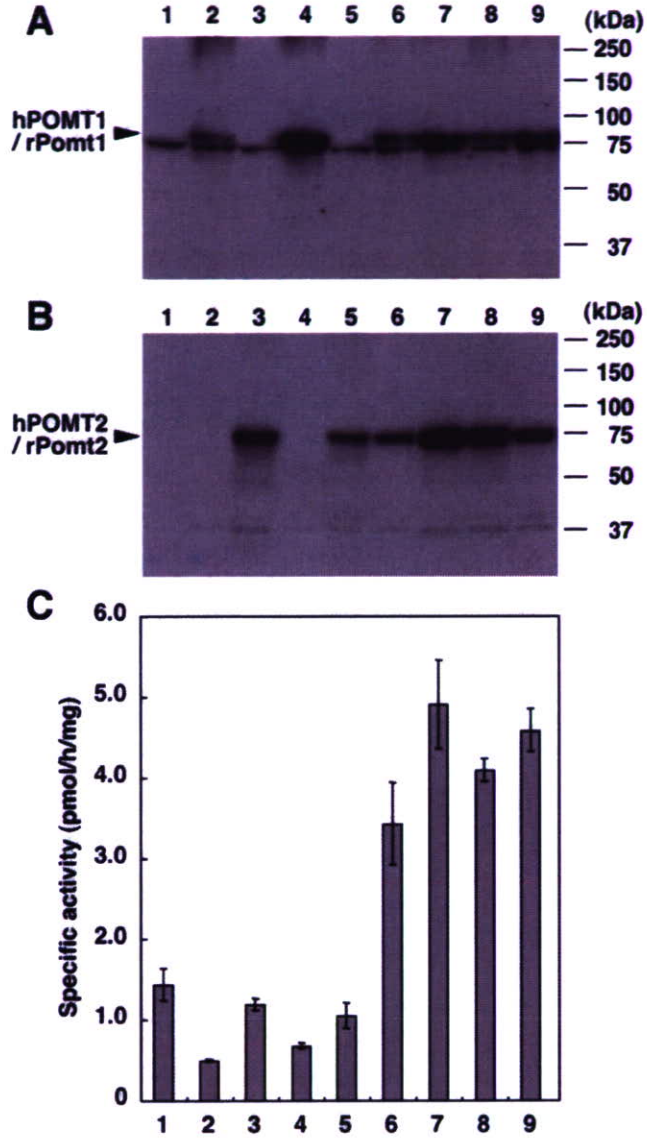

Fig. 6. Expression and activity of rat Pomt1 and s-Pomt2. (**A**, **B**) Western blot analyses of rat Pomt1, rat Pomt2, human POMT1, and human POMT2 expressed in HEK293T cells. The proteins (20 μg of microsomal membrane fraction) were subjected to SDS–PAGE (10% gel), and the separated proteins were transferred to a PVDF membrane. The PVDF membrane was stained with anti-POMT1 (**A**) or anti-POMT2 antibody (**B**). Molecular weight standards are shown on the right. (**C**) Protein *O*-mannosyltransferase activities in several combination conditions of rat and human POMTs. Protein *O*-mannosyltransferase activity was based on the rate of mannose transfer from Dol-P-Man to a GST-αDG. Lanes 1, cells transfected with vector alone; lanes 2, cells transfected with rat *Pomt1*; lanes 3, cells transfected with rat *s-Pomt2*; lanes 4, cells transfected with human *POMT1*; lanes 5, cells transfected with human *POMT2*; lanes 6, cells cotransfected with rat *Pomt1* and rat *s-Pomt2*; lanes 7, cells cotransfected with human *POMT1* and human *POMT2*; lanes 8, cells cotransfected with rat *Pomt1* and human *POMT2*; lanes 9, cells cotransfected with human *POMT1* and rat *s-Pomt2*. Average values of three independent experiments are shown.

not affect protein *O*-mannosyltransferase activity. A testis-specific form of Pomt2 translation was also observed in mouse (Willer *et al.*, 2002). In mice, the testis form of Pomt2 was localized to maturing spermatids and was distributed within the acrosome and the endoplasmic reticulum (ER). The authors of that study speculated that the ER-localized Pomt2 is involved in the synthesis of *O*-mannosyl glycans, and the acrosome-localized Pomt2 acts as a lectin that is involved in adhesive interactions of sperm and egg during fertilization (Willer *et al.*, 2002). In Figure 3A (middle panel), a band corresponding to the 2.7 kb mRNA in testis is thought to be the *s-Pomt2*. This conclusion is consistent with data from the mouse, insofar as mRNAs of both *s-Pomt2* and *t-Pomt2* were detected in mouse testis (Willer *et al.*, 2002). In Figure 7B, the band was detected around the migration position of s-Pomt2 at 75 kDa in the cells transfected with t-Pomt2 (lanes 2 and 4). Because the *t-Pomt2* cDNA has two ATG-start sites, it is likely that the 75-kDa band of t-Pomt2 is derived from the transcription starting at the second ATG-start site. The biological significance of the presence of different *Pomt2s* remains to be determined.

Why protein *O*-mannosyltransferase activity requires coexpression of Pomt1 and Pomt2 is unclear. One possibility is that Pomt1 is a catalytic molecule and Pomt2 is a regulatory molecule or *vice versa*. Another possibility is that assembly of Pomt1 and Pomt2 forms a catalytic domain. That is the reason why expression of Pomt1 or Pomt2 alone does not show any enzymatic activity. Recently, it has been reported that complex formation between a glycosyltransferase and

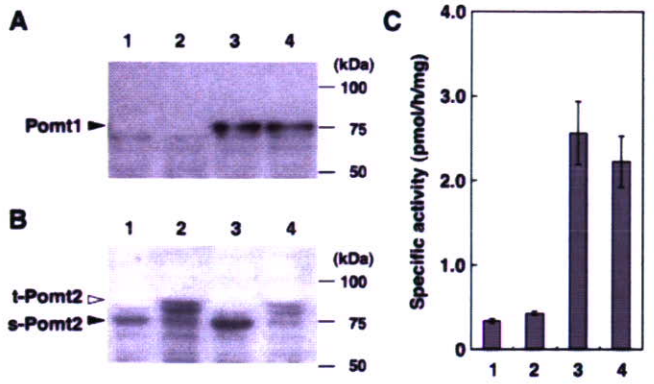

Fig. 7. Protein *O*-mannosyltransferase activity of testis form of rat Pomt2. (**A**, **B**) Western blot analyses of recombinant Pomt1 and Pomt2 detected by anti-POMT1 (**A**) and anti-POMT2 antibody (**B**). Molecular weight standards are shown on the right. (**C**) Protein *O*-mannosyltransferase activities of s-Pomt2 and t-Pomt2 coexpressed with Pomt1. Lanes 1, cells transfected with *s-Pomt2*; lanes 2, cells transfected with *t-Pomt2*; lanes 3, cells cotransfected with *Pomt1* and *s-Pomt2*; lanes 4, cells cotransfected with *Pomt1* and *t-Pomt2*. Average values of three independent experiments are shown.

its homologue changed its enzymatic character. Human chondroitin synthase exhibits glucuronyltransferase II and *N*-acetylgalactosaminyltransferase II activities but cannot polymerize the chondroitin chain *in vitro* (Kitagawa *et al.*, 2001). A recent study indicated that chondroitin-polymerizing activity requires the coexpression of chondroitin-polymerizing factor with chondroitin synthase (Kitagawa *et al.*, 2003). Although the amino acid sequence of chondroitin-polymerizing factor displayed 23% identity to that of chondroitin synthase, chondroitin-polymerizing factor did not show any enzymatic activity. Heparan sulfate polymerization is another case. Heparan sulfate polymerization *in vitro* requires both EXT1 and EXT2 that have *N*-acetylglucosaminyltransferase II and glucuronyltransferase II activities. A heterocomplex formation of EXT1 and EXT2 is required for chain elongation of heparan sulfate and to be present in the appropriate intracellular locations (McCormick *et al.*, 2000; Kim *et al.*, 2003). Further studies are needed to understand the regulation of protein *O*-mannosylation by two *Pomt* homologues.

Mutations in the human *POMT1* and *POMT2* genes give rise to WWS, a congenital muscular dystrophy with severe neuronal migration disorder (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). Elucidating the regulation of *O*-mannosylation in brain will help to understand the molecular pathology of WWS. To address the pathogenesis of WWS, it is also important to determine what proteins in addition to α-DG may be modified by *O*-mannosylation. The mannosyl-*O*-Ser/Thr linkage was first identified in chondroitin sulfate proteoglycans, neurocan, phosphacan, and phosphacan-keratan sulfate of brain (Finne *et al.*, 1979; Krusius *et al.*, 1986, 1987; Margolis *et al.*, 1996). The content of mannosyl-*O*-Ser/Thr linkage in these proteoglycans is regulated developmentally. On the basis of the yield of mannitol in hydrolysates of oligosaccharides after alkaline borohydride treatment of neurocan, the proportion of mannosyl-*O*-Ser/Thr linkage was calculated to increase from an insignificant level in one week postnatal rat brain to 15% of the total mannose in adult brain, whereas in the cases of phosphacan and phosphacan–keratan sulfate, the corresponding values were 26–31% at 7 days postnatal and 28–52% in adult brain (Rauch *et al.*, 1991). On the other hand, *N*-acetylgalactosamine (GalNAc)-linked oligosaccharides disappeared from phosphacan during the course of postnatal brain development, and these were replaced in adult brain by a significant proportion of oligosaccharides and keratan sulfate chains containing mannosyl-*O*-Ser/Thr linkages (Rauch *et al.*, 1991). If *O*-mannosylation and *O*-GalNAcylation occur on the same Ser/Thr residues of these proteoglycans, a developmental change of *O*-mannosylation may affect *O*-GalNAcylation, because *O*-mannosylation occurs in the ER and *O*-GalNAcylation takes place at a later processing step in the Golgi apparatus (Rottger *et al.*, 1998). Elucidating the regulation of *O*-mannosylation should therefore help in understanding the developmental roles of *O*-glycosylation in brain.

Materials and Methods

Cloning of rat Pomt1

Two degenerate oligonucleotide primers were designed on the basis of the amino acid sequences of *Saccharomyces cerevisiae* PMT1 corresponding to amino acids 486–496 and 652–662: 5′-cctcctcgagtggggitt(c/t)i(a/g)ica(a/g)caiga(a/g)gt-3′ (sense) and 5′-cgaactcgagigciggia(a/g)(a/g)ta(a/g)t(g/t)(a/g)t(a/g)ia(a/g)(a/g)aa-3′ (antisense) (XhoI sites are underscored). mRNA was isolated from adult Sprague–Dawley rat brain using the FastTrack kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's directions, and RT–PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer, Wellesley, MA). Amplified products were ligated into pBluescript SK(-) (Stratagene, La Jolla, CA) after XhoI digestion. One clone with a high-sequence identity to the *S. cerevisiae PMT*s and the *PMT* homologue of *Drosophila melanogaster* was obtained. Primers were designed on the basis of the sequence: 5′-ttcatggctctgccatagcggtg-3′ (antisense) and 5′-tggctggagccctgtgtattgg-3′ (sense). Using a combination of these primers and the Bluescript forward and reverse (BSK and BKS) primers to which we added six bases in each case, 5′ and 3′ portions of the clone were extended by PCR using a 6-week rat brain λZAPII cDNA library (Stratagene) as template. Amplified products were ligated directly into pGEM-7Z (Promega, Madison, WI). One clone was obtained for each side, and a sequence from 3′-extended one was utilized as a probe for cDNA library screening. The 854-bp cDNA fragment amplified by PCR was labeled by random priming with [α-^{32}P]dCTP, and using this radiolabeled probe, the 6-week rat brain λZAPII cDNA library was screened by plaque hybridization. After the second hybridization using the same probe, positive plaques were excised *in vivo* according to the manufacturer's instructions and sequenced.

To obtain further 5′-cDNA sequence, 5′-rapid amplification of cDNA ends (RACE) was carried out with a GeneAmp RNA PCR kit, terminal deoxynucleotidyl transferase (Promega) and *Taq* DNA polymerase (Perkin Elmer). The

specific antisense primers were 5′-catcgaatcctcctagccaacc-3′ comprising the nucleotides 377–356 for the reverse transcription and 5′-gtggtccactgtcatccaggaag-3′ comprising the nucleotides 326–304 for the nested PCR. The sense primers with linker were 5′-tggaagaattcgcggccgcagttttttttttttttt-3′ and 5′-tggaagaattcgcggccgcag-3′ (EcoRI sites are underscored). The amplified products were ligated into the pGEM-7Z vector and sequenced.

Cloning of rat Pomt2

Using a cDNA sequence of human *POMT2* as probe, we identified a sequence (accession number XM_345708.1) that appeared likely to encode a part of rat Pomt2 by BLAST search in the GenBank database. On the basis of this sequence, we obtained further 5′- and 3′-cDNA sequences of somatic form *Pomt2* (*s-Pomt2*) by RT–PCR from rat testis poly(A)$^+$ RNA (BD Biosciences, Franklin Lakes, NJ) using a SMART RACE cDNA Amplification Kit (BD Biosciences), according to the manufacturer's instructions. The specific primers for 5′-RACE and 3′-RACE were 5′-agccccacttaggcagaatc-3′ and 5′-agtctatctgctcggcaacc-3′, respectively. To obtain more of the 5′-cDNA sequence of the testis form *Pomt2* (*t-Pomt2*), RT–PCR was carried out with SuperScript III RNase H$^-$ Reverse Transcriptase (Invitrogen) and KOD-Plus-DNA polymerase (Toyobo Co., Osaka, Japan) using 5′-acagctcttctccgcgtttc-3′ for the sense primer, based on the cDNA sequence of mouse *Pomt2* (NM_153415), and 5′-aaagtcgggcttgaaaggca-3′ for the antisense primer, based on rat *s-Pomt2* mentioned above. The amplified cDNAs were cloned into pCR4Blunt-TOPO (Invitrogen) and sequenced.

Vector construction of rat Pomt1 and Pomt2

The *Pomt1* fragment in the pGEM-7Z vector was digested and then introduced into the EcoRI sites of the pcDNA3.1 vector (Invitrogen) to express Pomt1. To make expression plasmid vectors of *s-* and *t-Pomt2*, the cDNAs containing putative open reading frame (ORF) of *s-* and *t-Pomt2* were amplified by PCR using cloned cDNAs in pCR4Blunt-TOPO as template. Primers of *s-Pomt2* were 5′-ttaagcttgccaccatgccgccggccataggc-3′ and 5′-cactcgagtcaaaagtcccatgattcc-3′ (HindIII and XhoI sites are underscored). Primers of *t-Pomt2* were 5′-ctaagcttcctgctatgttttacgcctc-3′ and 5′-gctgtggtgctcgtatcggt-3′ (HindIII site is underscored). The PCR product of *s-Pomt2* was cloned into the HindIII and XhoI sites of pcDNA3.1 vector (pcDNA3.1-*s-Pomt2*), and the PCR product of *t-Pomt2* was introduced into the HindIII and KpnI sites of pcDNA3.1-*s-Pomt2*. Human *POMT1* and *POMT2* cDNAs were cloned into pcDNA3.1 as described previously (Manya *et al.*, 2004).

Nucleotide sequence and protein sequence analyses

Protein sequences were aligned and placed in a phylogenetic tree with ClustalW (http://www.ddbj.nig.ac.jp/searches-e.html). Similarities and identities were analyzed using the GENETYX-Mac program (GENETYX Corp., Tokyo, Japan), based on Lipman-Pearson's method, and the gap was not counted.

Northern blot analysis

Northern blots of rat tissues (Rat MTN blot) were purchased from BD Biosciences. Probe DNA fragments for rat *Pomt1* and *Pomt2* were prepared by PCR, and β-actin was supplied with the Rat MTN blot. Primers for *Pomt1* were 5′-cccactgtactgccatggg-3′ and 5′-ccatgtctcacaggcctg-3′. Primers for *Pomt2* were 5′-cctgggttcctggctgatc-3′ and 5′-cggattacaa gtaacttccaactgc-3′. Each probe was labeled with [α-^{32}P] dCTP using a Random Primer DNA labeling kit (Takara Bio Inc., Shiga, Japan). Blots were hybridized with a ^{32}P-labeled DNA probe in ExpressHyb Hybridization solution (BD Biosciences) at 68°C for 1 h, followed by washing according to the manufacturer's instruction.

RT–PCR analysis

First-strand cDNAs were synthesized from poly(A)$^+$ RNAs of rat tissues (Rat MTC Panel I; BD Biosciences) using SuperScript III RNase H$^-$ Reverse Transcriptase. PCR was carried out with KOD-Plus-DNA polymerase using the following primers: 5′-acagctcttctccgcgtttc-3′ and 5′-gtagccagcaaggccaatca-3′ for the testis-specific sequence of *Pomt2* (841 bp); 5′-gagacattgtacagctcgtt-3′ and 5′-ctggctgaagtccagatcac-3′ for *Pomt1* (618 bp); and 5′-taatcactgtgaagaacctc-3′ and 5′-gaattcgacttctcagtacc-3′ for *Pomt2* (399 bp). Primers for rat glyceraldehyde 3-phosphate dehydrogenase (Rat G3PDH Control Amplimer Set) were purchased from BD Biosciences. The cycling parameters for PCR were 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min, and cycle numbers were 40 cycles for testis-specific sequence of *Pomt2* and 35 cycles for *Pomt1*, *Pomt2*, and *G3PDH*.

In situ *hybridization histochemistry*

An EcoRI-XbaI restriction fragment of *Pomt1* comprising 331 nucleotides (nucleotides 2424–2754) and an EcoRI-PstI restriction fragment of *Pomt2* comprising 463 nucleotides (nucleotides 1902–2364) were subcloned into the pGEM-3Z vector (Promega), and the vectors were linearized with EcoRI for antisense probes of *Pomt1* and *Pomt2*, XbaI for sense probe of *Pomt1*, or PstI for sense probe of Pomt2. For PstI digestion, the linearized template end was converted to a blunt end with T4 DNA polymerase (Promega). Digoxigenin-labeled RNA probes were synthesized with the Riboprobe System (Promega) and digoxigenin-11-dUTP (Roche Diagnostics, Tokyo, Japan). To detect the expression of *Pomt1* and *Pomt2* in brain, frozen sections of rat brain tissues were fixed with 3% paraformardehyde/10 mM phosphate-buffered saline (PBS) and then analyzed with an In situ Hybridization kit (NIPPON GENE, Tokyo, Japan) according to the manufacturer's directions except that the sections were washed at 48°C after hybridization.

Expression of POMTs and cell extract preparation

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, and 100 units/mL penicillin–50 mg/mL streptomycin at 37°C with 5% CO_2. The expression plasmids of pcDNA3.1-rat *Pomt1*, pcDNA3.1-rat *s-Pomt2*, pcDNA3.1-rat *t-Pomt2*, pcDNA3.1-human *POMT1-myc*,

and pcDNA3.1-human *POMT2* were transfected into HEK293T cells using LipofectAMIN PLUS reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were cultured for 3 days in complete medium, harvested and homogenized. The cells were homogenized in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, with a protease inhibitor cocktail (3 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 mM benzamidine–HCl, and 1 mM PMSF). After centrifugation at 900 × *g* for 10 min, the supernatant was subjected to ultra centrifugation at 100,000 × *g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay (PIERCE, Rockford, IL).

Preparation of rat tissues

Heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis were obtained from 4-month-old Wistar rats. Tissue samples were homogenized with nine volumes (weight/volume) of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 250 mM sucrose. After centrifugation at 900 × *g* for 10 min, the supernatant was subjected to ultra centrifugation at 100,000 × *g* for 1 h. The precipitates were used as the microsomal membrane fraction. Protein concentration was determined by BCA assay. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology. All efforts were made to minimize the number of animals used and their suffering.

Western blot analysis

Rabbit antibodies specific to POMT1 and POMT2 were described previously (Manya *et al.*, 2004). The microsomal fractions (20 μg) were separated by SDS–PAGE (10% gel), and proteins were transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with each antibody, and treated with anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ). Proteins bound to antibody were visualized with an ECL kit (Amersham Biosciences). As reported previously (Manya *et al.*, 2004), anti-POMT1 and anti-POMT2 polyclonal antibodies did not detect endogenous POMT1 and POMT2, respectively. Each antibody is specific for the respective recombinant protein.

Assay for protein O-mannosyltransferase activity

Protein *O*-mannosyltransferase activity was based on the amount of [^{3}H]-mannose transferred from Dol-P-Man to a glutathione-S-transferase fusion α-DG (GST-αDG) as described previously (Manya *et al.*, 2004). Briefly, the reaction mixture contained 20 mM Tris–HCl (pH 8.0), 100 nM of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemicals Inc., St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg GST-αDG, and enzyme source (80 μg of microsomal membrane fraction) in 20 μL total volume. After 1 h incubation at 25°C, the reaction was stopped by adding 150 μL PBS containing 1% Triton X-100 (Nacalai Tesque, Kyoto, Japan), and the reaction mixture was centrifuged at 10,000 × *g* for 10 min. The supernatant was removed, mixed with 400 μL of PBS containing 1% Triton X-100 and 10 μL of Glutathione-Sepharose 4B beads (Amersham Biosciences), rotated at 4°C for 1 h, and washed three times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured with a liquid scintillation counter.

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Conflict of interest statement

None declared.

Abbreviations

α-DG, α-dystroglycan; Dol-P-Man, dolichyl phosphate mannose; ER, endoplasmic reticulum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GalNAc, *N*-acetylgalactosamine; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcriptase polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; WWS, Walker–Warburg syndrome.

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Regulation of Mammalian Protein *O*-Mannosylation

PREFERENTIAL AMINO ACID SEQUENCE FOR O-MANNOSE MODIFICATION[*] [S]

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Hiroshi Manya[‡], Takehiro Suzuki[§¶], Keiko Akasaka-Manya[‡], Hide-Ki Ishida[‖], Mamoru Mizuno[**], Yasushi Suzuki[‡‡], Toshiyuki Inazu[‡‡], Naoshi Dohmae[§¶], and Tamao Endo[‡1]

From the [‡]Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, Itabashi-ku, Tokyo 173-0015, [§]Biomolecular Characterization Team, RIKEN, Wako-shi, Saitama 351-0198, [¶]Japan Science and Technology Agency CREST, [‖]Tokyo Chemical Industry Co., Ltd., Kita-ku, Tokyo 114-0003, [**]Research Department, The Noguchi Institute, Itabashi-ku, Tokyo 173-0003, and [‡‡]Department of Applied Chemistry, School of Engineering and Institute of Glycotechnology, Tokai University, Hiratsuka-shi, Kanagawa 259-1292, Japan

O-Mannosyl glycans are important in muscle and brain development. Protein *O*-mannosyltransferase (POMT) catalyzes the initial step of *O*-mannosyl glycan biosynthesis. To understand which serine (Ser) and threonine (Thr) residues POMT recognizes for mannosylation, we prepared a series of synthetic peptides based on a mucin-like domain in α-dystroglycan (α-DG), one of the best known *O*-mannosylated proteins in mammals. In α-DG, the mucin-like domain spans amino acid residues 316 to 489. Two similar peptide sequences, corresponding to residues 401–420 and 336–355, respectively, were strongly mannosylated by POMT, whereas other peptides from α-DG and peptides of various mucin tandem repeat regions were poorly mannosylated. Peptides 401–420 and 336–355 contained four and six Ser and Thr residues, respectively. Substitution of Ala residues for the Ser or Thr residues showed that Thr-414 of peptide 401–420 and Thr-351 of peptide 336–355 were prominently modified by *O*-mannosylation. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry and Edman degradation analysis of the mannosylated peptide 401–420 indicated that Thr-414 was the Thr residue that was most prominently modified by *O*-mannosylation and that *O*-mannosylation occurred sequentially rather than at random. Based on these results, we propose a preferred amino acid sequence for mammalian *O*-mannose modification.

O-Mannosyl glycans are important in muscle and brain development (1). We previously found that the glycans of α-dystroglycan (α-DG)[2] predominantly include *O*-mannosyl glycan Siaα2–3Galβ1–4GlcNAcβ1–2Man (2). α-DG is a component of the dystrophin-glycoprotein complex that acts as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton (3). Previously we reported that defects in *O*-mannosyl glycan cause a type of muscular dystrophy (4, 5). We have found that protein *O*-mannosyltransferase 1 (POMT1) and its homolog POMT2 are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis (6). Mutations in *POMT1* and *POMT2* genes are considered to be the cause of Walker-Warburg syndrome (WWS: OMIM 236670), an autosomal recessive developmental disorder associated with congenital muscular dystrophy, neuronal migration defects, and ocular abnormalities (7, 8). We have demonstrated that mutations in the *POMT1* gene abolish POMT activity (9, 10). Thus, *O*-mannosylation is indispensable for normal structure and function of α-DG in muscle and brain in human.

We recently demonstrated that formation of a POMT1-POMT2 complex was required for POMT activity (10). POMT1 and POMT2 are homologous to members of the family of protein *O*-mannosyltransferases (PMTs) in yeast. PMTs were shown to catalyze the transfer of a mannosyl residue from dolichyl phosphate mannose to Ser/Thr residues of certain proteins (11). Individual PMTs have different specificities for protein substrates (12, 13), suggesting the presence of some sequence for recognition by PMTs, but the sequence was not identified. On the other hand, in mammals, *O*-mannosylated proteins are rare and *O*-mannosylation may require a specific sequence because we detected POMT activity when a glutathione *S*-transferase-fused mucin-like domain of α-DG (amino acid residues 316–489) was used as an acceptor (6). Previous studies suggested that the mucin-like domain is highly glycosylated and that certain glycans of α-DG play an important role in its binding to ligand proteins such as laminin, neurexin, and agrin (2, 14–17). To address the biological function and regulation of *O*-mannosylation, it is important to determine whether

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[1] To whom correspondence should be addressed: Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015, Japan. Tel.: 81-3-3964-3241 (ext. 3080); Fax: 81-3-3579-4776; E-mail: endo@tmig.or.jp.

[2] The abbreviations used are: α-DG, α-dystroglycan; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PMT and POMT, protein *O*-mannosyltransferase; WWS, Walker-Warburg syndrome; Con A, concanavalin A; HPLC, high pressure liquid chromatography.

there is a preferential amino acid sequence. In this study we synthesized a series of peptides that fully covered the mucin-like domain of α-DG (Fig. 1). Then we examined whether these peptides worked as acceptors for protein *O*-mannosylation. Further, the enzymatic products were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Edman degradation to determine the number of mannose residues transferred and the sites to which they were transferred. Based on the results of these studies, we proposed a preferred sequence for mammalian *O*-mannosylation.

EXPERIMENTAL PROCEDURES

Chemicals—Synthetic peptides were purchased by made-to-order system on the web site of Sigma-Aldrich (www.genosys.jp, Tokyo, Japan), and the quality of the synthesis was ascertained by HPLC analysis and mass spectrometry. A series of peptides, whose average length is ~20 amino acids, fully covered the mucin-like domain of α-DG (Table 1). Triton X-100 was purchased from Nacalai Tesque (Kyoto, Japan). *n*-Octyl-β-D-thioglucoside was from Dojindo Laboratories (Kumamoto, Japan). Tritium-labeled or unlabeled mannosylphosphoryldolichol were purchased from American Radiolabeled Chemical, Inc. (St. Louis, MO).

Mannosyl-threonine was synthesized as follows. To a solution of phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-α-D-mannopyranoside (1.0 g, 1.6 mmol) and *N*-benzyloxycarbonyl-L-threonine benzyl ester (813 mg, 2.4 mmol) in toluene (30 ml) and CH_2Cl_2 (10 ml), molecular sieves 4A (2.0 g) was added, and the mixture was stirred for 1 h at room temperature and then cooled to 0 °C. The cooled mixture were added to *N*-iodosuccinimide (720 mg, 3.2 mmol) and trifluoromethanesulfonic acid (30 μl, 0.32 mmol), and the mixture was further stirred for 2 h at 0 °C. The precipitates were filtered off through Celite and washed with CH_2Cl_2. Combined filtrate and washings were washed with saturated Na_2CO_3 and saturated $Na_2S_2O_3$, dried, and concentrated. Column chromatography of the residue on silica gel (AcOEt:Hexane = 1:4) gave *O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-*N*-benzyloxycarbonyl-L-threonine benzyl ester (1.05 g, 77%). To a solution of *O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-*N*-benzyloxycarbonyl-L-threonine benzyl ester (500 mg, 0.58 mmol) in MeOH (50 ml) 10% Pd on activated carbon (500 mg) and acetic acid (50 μl) were added. H_2 gas was bubbled into the mixture, which was stirred for 2 h at room temperature. The precipitates were filtered off and washed with water. The filtrate and washings were combined and concentrated to solid, which was chromatographed on Sephadex G-50 (eluent: water) to give *O*-(α-D-mannopyranosyl)-L-threonine (170 mg, quantitative). The structure was identified by ^{1}H-NMR and MALDI-TOF MS.

Mannosyl peptide 401–420(T414Man) (IRPTMTIPGYVEPT(Man)AVATPP) was synthesized essentially as described in our previous report (18). The structure was identified by ^{1}H-NMR and MALDI-TOF MS.

POMT Enzyme Source—The microsomal membrane fraction of human embryonic kidney 293T cells co-transfected by *POMT1* and *POMT2* was used for POMT enzyme source. A cDNA containing the most common splicing variant of human *POMT1* (which lacks bases 700–765, corresponding to amino acids 234–255) and human *POMT2* cDNA were cloned into pcDNA 3.1 (Invitrogen) as described previously (6). Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C with 5% CO_2. Expression plasmids were transfected into human embryonic kidney 293T cells using Lipofectamine plus regent (Invitrogen) according to the manufacturer's instructions. The cells were incubated for 3 days at 37 °C to produce POMT1 and POMT2 proteins. The cells were homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol with a protease inhibitor mixture (3 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 900 × *g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000 × *g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay.

Assay for POMT Activity—POMT activity was based on the amount of [^{3}H]mannose transferred from mannosylphosphoryldolichol to synthetic peptides. The reaction mixture containing 20 mM Tris-HCl, pH 8.0, 100 nM mannosylphosphoryldolichol (125,000 dpm/pmol), 2 mM dithiothreitol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 0.25–4 mM synthetic peptide; 40 μg of microsomal membrane fraction in 20 μl of total volume was incubated for 60 min at 25 °C. After boiling for 3 min, the mixture was analyzed by reversed-phase HPLC with a Wakopak 5C18–200 column (4.6 × 250 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or a Mightysil RP-18GP Aqua column (4.6 × 250 mm; Kanto Chemical Co., Inc., Tokyo, Japan). Solvent A was 0.1% trifluoroacetic acid in distilled water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–50% solvent B. The peptide separation was monitored continuously at 215 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

Peptide Sequencing and Mass Spectrometry—A mannosylated peptide 401–420 was prepared using concanavalin A (Con A)-Sepharose 4B beads (GE Healthcare) as follows. The reaction mixture containing 20 mM Tris-HCl, pH 8.0, 300 μM unlabeled mannosylphosphoryldolichol, 2 mM dithiothreitol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 1 mM peptide 401–420; 120 μg of microsomal membrane fraction in 30 μl of total volume was incubated for 15–120 min at 25 °C. The reaction mixture was mixed with 400 μl of 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ and centrifuged at 10,000 × *g* for 10 min. The supernatant was loaded onto the Con A-Sepharose column (100 μl of bed volume) and washed with 2 ml of the same buffer. The bound fraction was obtained by eluting with 250 μl of 200 mM α-methylmannoside in 20 mM Tris-HCL, pH 7.4, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ and separated by reversed-phase HPLC as described above.

Con A-purified mannosyl peptide preparation was fractionated by reversed-phase HPLC on an Inertsil ODS-3 column